

Multiple End Point Procedure to Evaluate Risk from Pesticides

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Because of the potential environmental impact of pesticides and the large population potentially exposed, the effects of chronic exposure to pesticides need to be determined. Mutagenicity studies have been used to identify specific agents as potential carcinogens or other human health hazards. However, short-term tests are only theoretically correlated to carcinogenesis because their end points can measure only the genotoxic potential of chemicals, i.e., their activities as initiating agents in multistep carcinogenesis. The objective of our research presented here is to provide a comprehensive examination of the mechanism of toxicity of a series of pesticides. These are substances for which toxicity, at both the genetic and metabolic level, has not been adequately described. Preliminary results on a broad series of compounds belonging to different biological classes (herbicide, insecticide, fungicide) seem to indicate that pesticides are toxic but are poor initiating agents, as shown by negative or weak positive results on different genetic end points (gene mutations, DNA effects, and chromosome aberrations *in vitro* and *in vivo*). Immunochemical and biochemical studies, however, seem to indicate the cocarcinogenic and promoting potential of these chemicals. As an example, the genotoxic and biochemical effects induced by Fenarimol (a fungicide) are discussed. The results reported stress the importance of identifying chemicals that act at different levels of the multistep carcinogenesis process to ascertain the risk associated with exposure.

Introduction

The broad use of pesticides represents a potential risk to humans and to the environment. A principal goal in pesticide research and development is identifying the specificity of action of a pesticide toward the organisms it is supposed to kill. Only the target plant, animal, insect or fungus should be affected by the application of the product. However, because pesticides are designed and selected for their biological activity, toxicity to nontarget species usually remains a significant potential problem (1).

Unintended exposure to pesticides can occur during manufacture, formulation, application, or from environmental residues after application. Because each type of exposure has its own characteristics of magnitude and duration and may lead to different toxicity, it is important to consider efficacy and safety simultaneously. One of the factors underlying the increased concern about the health hazards associated with the use of pesticides is a growing awareness of the hazards associated with long-term exposure to some of these chemicals that are persistent in soil (organochlorine and carbamate insecticides and certain herbicides). The insidious nature of effects such as mutagenicity and carcinogenicity has drawn the attention of scientists and government agencies. More comprehensive

safety testing is required before a new pesticide is registered for use, and major efforts to reexamine the safety data available for chemicals already in use are underway within regulatory agencies (2). Predicting potential human risk from pesticides is based on a wide array of toxicological studies, from acute toxicity to long-term effects, but risk assessment is especially complex for chronic disease outcomes such as cancer, as recently stressed by Legator and Ward (3).

Carcinogenic Assessment of Pesticides

Testing chemicals for possible carcinogenic effects involves many approaches. The use of short-term mutagenicity bioassays, aimed at identifying genotoxic carcinogens at an early stage, overcomes the high costs and long time associated with animal carcinogenicity bioassays. Many sophisticated short-term tests for determining genotoxicity have been developed, but they are used primarily for screening for genetic toxicity. Short-term tests cannot completely replace long-term bioassays because the correlation between genotoxicity and carcinogenicity is not as direct as was originally hypothesized [see for example Zeiger (4,5) and Tennant (6)].

Short-term tests mimic only a part of the phenomena that would occur *in vivo* (7). Cancer is now known to be a multifactorial, multistage, and multimechanistic disease in which many factors are relevant (8,9). When chemicals are

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shown to be mutagenic and induce cancer in a dose-dependent manner in animals, they are generally regarded as important carcinogens because they initiate the disease, as stipulated by the somatic mutation theory. However, recent research has shown that factors modifying the progression of the disease (promoters and inhibitors) may be considerably important as risk determinants (10,11).

Our approach to studying the potential risk from pesticides consists of the development of a database of toxicological information (general toxicology, mutagenesis, and carcinogenesis) on pesticides. Risk assessment from experimental studies is based on a comprehensive examination on the mechanism of toxicity at the genetic and metabolic (biochemical) levels. With regard to genotoxicity studies, particular attention is given to cytogenetic assays because chromosome aberration end points can be used as an early warning signal for development of cancer (12). Experimental results are further evaluated with a semi qualitative approach using categories of concern (2,3).

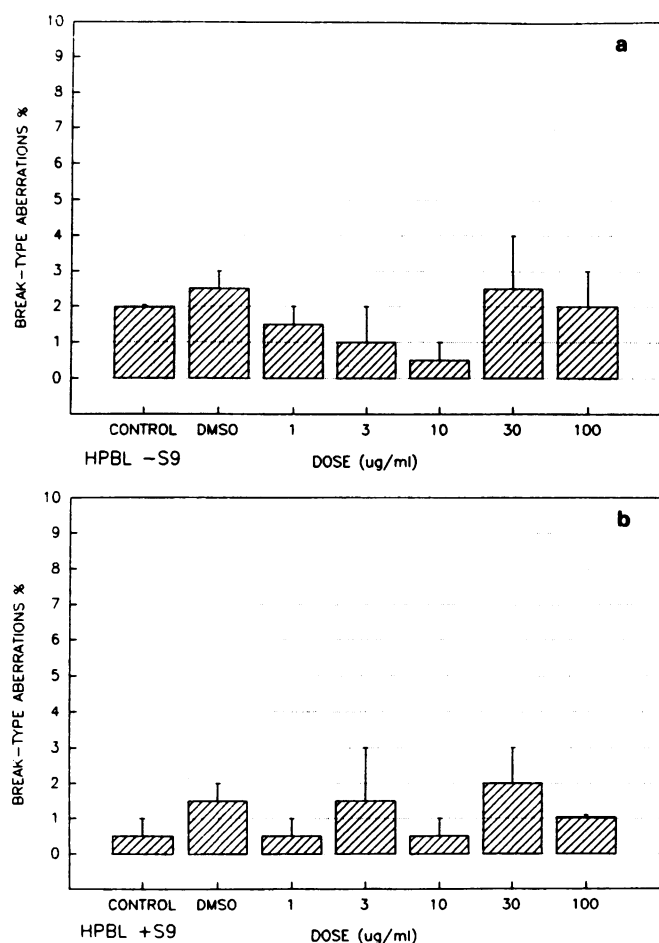


FIGURE 1. Incidence of break-type aberrations induced in human lymphocytes exposed *in vitro* to Fenarimol in absence (a) and presence (b) of S9 mix. Results are means \pm SD of data obtained from two different donors.

Preliminary results on a broad series of compounds, belonging to different biological classes (herbicides, insecticides, fungicides) seem to indicate that most pesticides are toxic but are poor initiating agents, as shown by negative or weak positive results at different genetic end points (13,14). Biochemical and immunochemical studies show that pesticides induce mixed-function oxidase enzymes (P-450IIB1) in different organs of rodents indicating a cotoxic, cocarcinogenic and promoting potential of these chemicals (Paolini and Cantelli-Forti, unpublished results).

The promoting properties of cytochrome P-450IIB1 subfamily of isozymes inducers (e.g., barbiturates, haliphatic halogenated hydrocarbons) are acknowledged. However, it should be noted that other inducers such as alcohols (P-450IIE1) and dioxins (P-450IA2) are promoters also (15,16).

Case Study: Fenarimol

Fenarimol [2,4-dichloro- α -(pyrimidin-5-yl)-benzhydryl alcohol] is a fungicide with protective, curative, and eradicated activities. A recent report of the U.S. National Research Council (17) stated that fungicides contribute nearly 60% of all estimated oncogenic risk from pesticides. The committee concluded that the mode of action of fungicides makes it rather difficult to develop compounds that do not damage genetic material (17).

The evidence in the literature for the examined fungicides is conflicting and incomplete. In most cases, literature data on Fenarimol consists of summary of data without references. This introduces considerable bias into any assessment of the available data. Fenarimol is reported to be negative for the induction of gene mutation

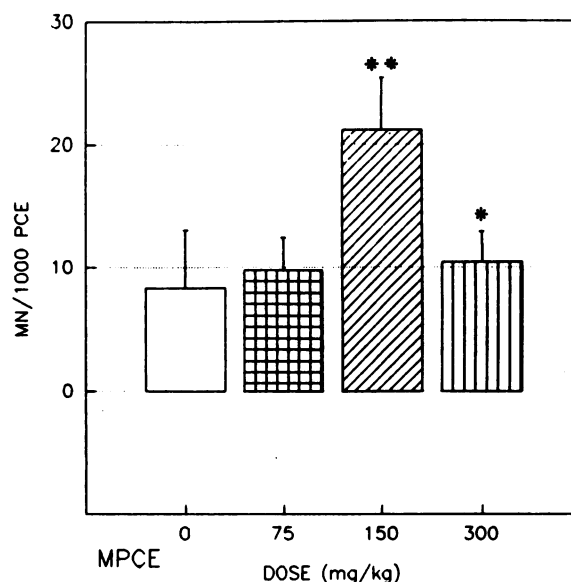


FIGURE 2. Frequency of micronucleated bone marrow erythrocytes in mice treated with different doses of Fenarimol. (**) $p < 0.01$, (*) $p < 0.05$ with respect to control, Chi-square test.

in bacteria and in mouse lymphoma cells at the *tk* locus (18,19). No induction of unscheduled DNA synthesis was evaluated in primary rat hepatocytes, whereas the agent was shown to cause mitotic nondisjunction in *Aspergillus nidulans* (20). Fenarimol has been associated with increased frequencies of micronuclei in bone marrow of mice after two repeated doses close to the LD₅₀ (18). This evidence is contrasted by negative results for induction of chromosome aberrations in Chinese hamster bone marrow and in the dominant-lethal mutagenicity assay in rat (18,19).

In the study done in our laboratories, Fenarimol was assessed for clastogenicity with two different assays, namely, frequency of chromosome aberrations in human

peripheral lymphocytes (21) and an assessment of the micronuclei frequency in mouse bone marrow using the acridine orange staining method (22,23). In particular, the micronucleus technique can measure effects on the spindle apparatus (aneugenicity) and chromosomes [clastogenicity/genotoxicity (24)].

Exposure of human lymphocytes *in vitro* obtained from two different donors to different doses of Fenarimol, ranging from 1 to 100 µg/mL both in the presence and the absence of a microsomal S9 fraction obtained from sodium phenobarbital- and β-naphthoflavone-induced rat liver, did not induce any significant increase in chromosome aberrations (Fig. 1) Otherwise, the treatment of male mice (Swiss

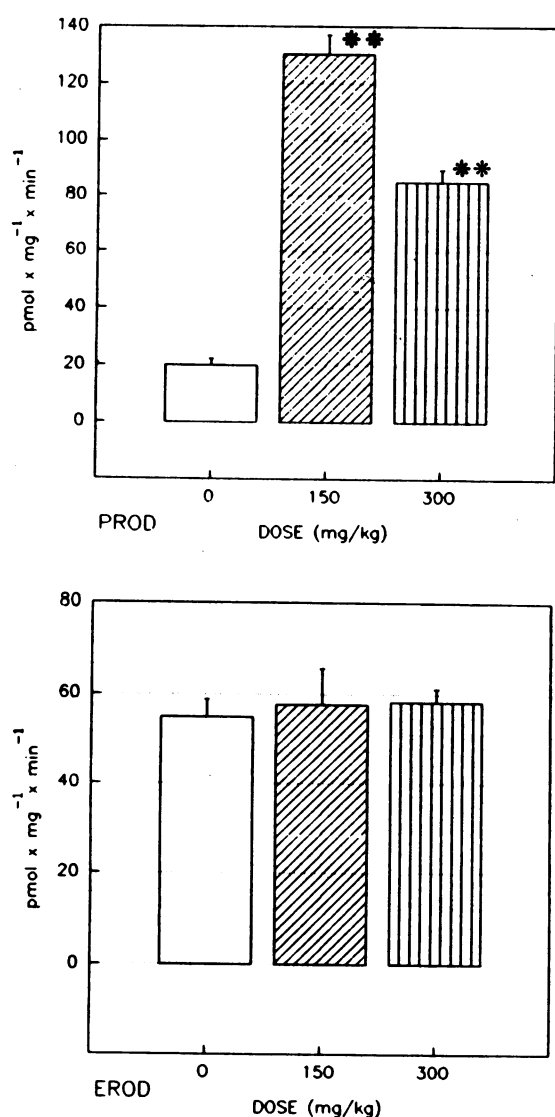


FIGURE 3. Expression of pentoxyresorufin *O*-dealkylase (PROD) activity and ethoxyresorufin *O*-deethylase (EROD) activity in liver microsomes from Fenarimol-intoxicated mice. (□) Corn oil (control), (▨) treated with Fenarimol, 150 mg/kg body weight, and (▩) 300 mg/kg body weight. Results are means ± SD of six independent experiments. (**) $p < 0.01$ with respect to control, using Wilcoxon's rank method.

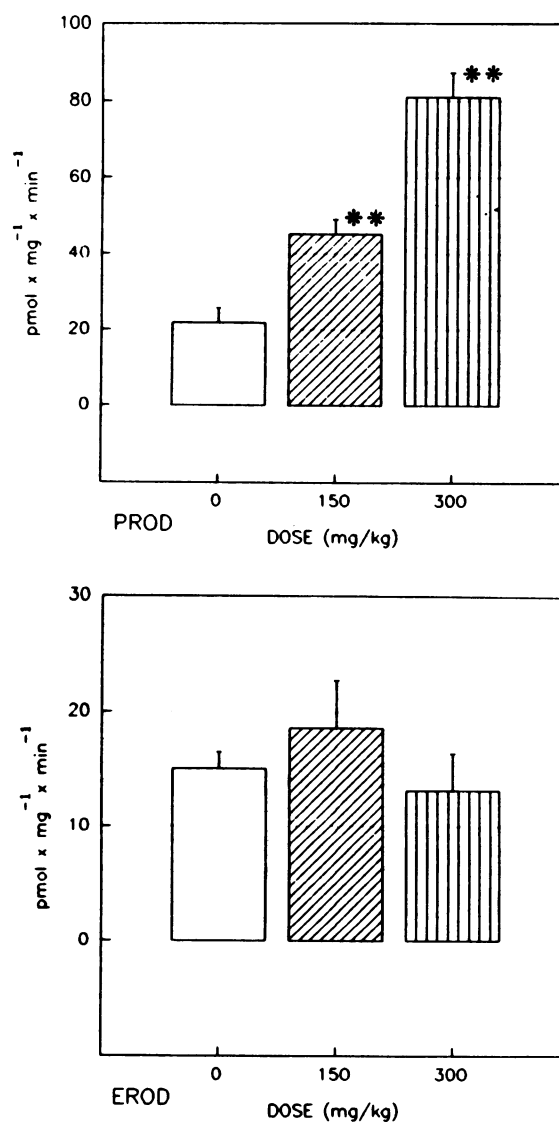


FIGURE 4. Expression of pentoxyresorufin *O*-dealkylase (PROD) activity and ethoxyresorufin *O*-deethylase (EROD) activity in kidney microsomes from Fenarimol-intoxicated mice. (□) Corn oil (control), (▨) treated with Fenarimol, 150 mg/kg body weight, and (▩) 300 mg/kg body weight. Results are the means ± SD of six independent experiments. (**) $p < 0.01$ with respect to control, using Wilcoxon's rank method.

albino CD1 strain) with three different doses of Fenarimol, from 75 to 300 mg/kg body weight, resulted in significant increases of micronucleated erythrocytes (up to 5-fold over the control; $p < 0.01$), as detected in bone marrow smears (Fig. 2). The increases are dose related until a depression of erythropoiesis appeared at the higher dose, corresponding to the 50% of the LD_{50} , previously evaluated. The disagreement of results obtained in the two cytogenetic assays suggests that Fenarimol may act as an aneugen and not a clastogen. This hypothesis, supported by the reported effect of Fenarimol on distribution of chromosomes leading to mitotic nondisjunction in *Aspergillus nidulans* (20), needs to be validated by further experiments.

To ascertain the possible cotoxic, cocarcinogenic, and promoting properties of Fenarimol, the ability to modulate

specific P-450-dependent activities in the mouse was studied. For this purpose, the fungicide was administered IP at 150 or 300 mg/kg body weight in male mice (Swiss albino CD1 strain), and the purified microsomal fraction was then prepared from liver, kidney, and lung (25). NADPH-cytochrome (P-450)c-reductase activity and selected substrates (as probes) of specific P-450 isoenzymes, such as pentoxyresorufin (P-450IIB1), ethoxyresorufin (IA1), aminopyrine (IIIA), and ethoxycoumarin toward unspecific P-450 isoforms were used (25).

The dealkylation of pentoxyresorufin was significantly increased up to 6.9-fold at 150 mg/kg dose in liver (Fig. 3). On the contrary, no significant changes in the P-450IA1-dependent oxidases was observed (Fig. 3). A similar pattern of enzymatic activity was recorded in kidney and lung microsomes. An enhancement of IIB1-like activity up to

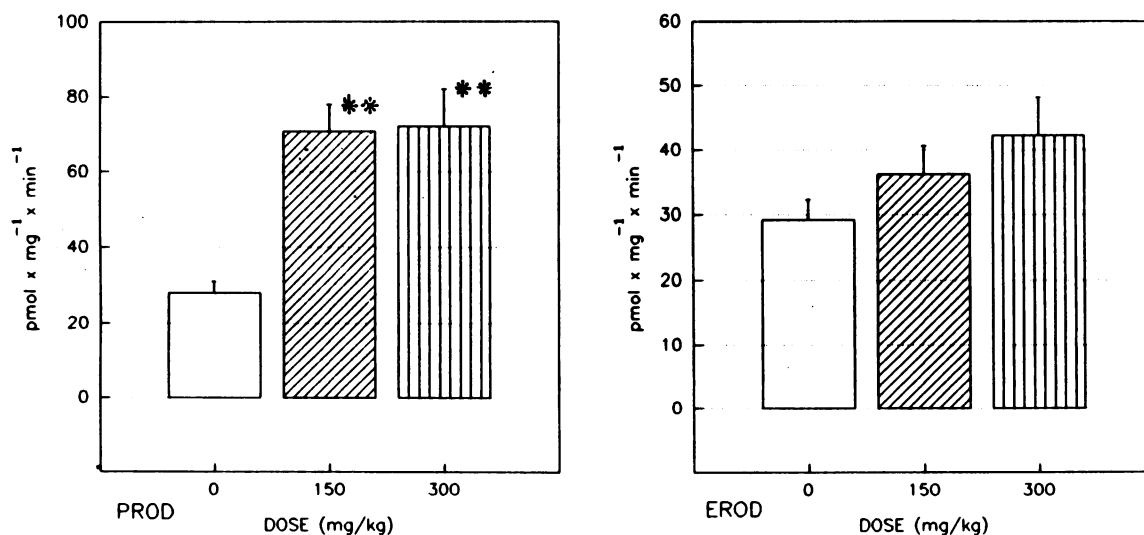


FIGURE 5. Expression of pentoxyresorufin *O*-dealkylase (PROD) activity and ethoxyresorufin *O*-deethylase (EROD) activity in lung microsomes from Fenarimol-intoxicated mice. (□) Corn oil (control), (▨) treated with Fenarimol, 150 mg/kg body weight, and (▩) 300 mg/kg body weight. Results are the means \pm SD of six independent experiments. (**) $p < 0.01$ with respect to control, using Wilcoxon's rank method.

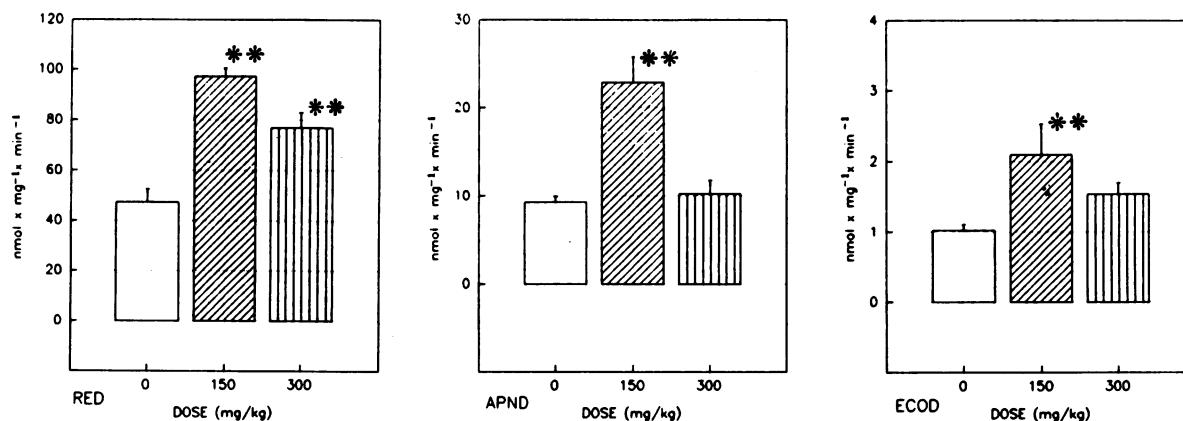


FIGURE 6. Expression of NADPH-cytochrome (P-450) *c*-reductase and oxidative reactions in hepatic microsomes from Fenarimol-intoxicated mice. (□) Corn oil (control), (▨) treated with Fenarimol, 150 mg/kg body weight, and (▩) 300 mg/kg body weight. Results are the means \pm SD of six independent experiments. (**) $p < 0.01$ with respect to control, using Wilcoxon's rank method.

2.2- and 3.8-fold in kidney (Fig. 4) and 2.5- and 2.6-fold in lung microsomes (Fig. 5) was indeed achieved at 150 and 300 mg/kg, respectively. Ethoxyresorufin *O*-deethylase was not significantly affected in both organs. The expression of IIA P-450-isoforms and ethoxycoumarin *O*-deethylase activity (mixed) was increased at 150 mg/kg dose only (Fig. 6). Finally, increase of reductase activity supports the monooxygenase inductions.

The induced expression of *Cyp2B1* genes in mouse after repeated administration (3 days) of Fenarimol was demonstrated by immunochemical analysis. Using rabbit polyclonal antibody against P-450IIB1 (anti-cyt-P-450IIB1 purified from rat liver), we obtained clear evidence of induction of constitutive levels of P-450IIB1 proteins that corroborate enzymatic results. The Western blotting assay, representative of a typical experiment in liver, is shown in Figure 7.

Conclusions

A principal goal in pesticide research is to design and develop agents with the maximum desired response and minimum deleterious side effects. This selectivity problem has become more acute, and the search of new biologically active compounds continues with increasing emphasis on safety. As stochastic effects such as genotoxicity and carcinogenicity are considered among the most serious of the possible adverse effects of pesticides, comprehensive safety testing is required for new molecules. A reexamination of the safety data available for agents already in use is of special concern in terms of health and environmental problems.

The literature of pesticides reveals great disparities in the extent of knowledge concerning specific mechanism of action. Most data come from short-term tests for genotoxicity concerning the initiating potency of a pesticide. Otherwise, little attention is given to the promoting and cocarcinogenic potential. The induction of metabolizing enzymes in the liver and elsewhere may in fact result in an altered metabolic state after long-term exposure.



FIGURE 7. Western blotting analysis of mouse microsomes using polyclonal antibody against cytochrome P-450IIB1. Position of purified antigen (M_r , 45,000) is indicated on the left ordinate. Untreated (corn oil, lane 1), treated with sodium phenobarbital (100 mg/kg body weight for 3 consecutive days, lane 2), and treated with Fenarimol (150 mg/kg body weight for 3 consecutive days, lane 3); 25 μ g of microsomal protein were electrophoresed in each lane.

The importance of this phenomenon has come from the consideration that, independent of the possible mutagenic (and/or teratogenic) property, a chemical inducing P-450 function can have serious toxicological and/or genetic consequences. In addition to the increase of oxidative, reductive, and peroxidative metabolism of pretoxics and premutagens/precarcinogens (cototoxicity and cocarcinogenicity), and the fact that an inducer can act as promoter in the multistep process of carcinogenesis (at least for classes IIB1, IA2, and IIE1 P-450s), P-450 induction per se can have a role in the development of malignancy (25,26). Indeed, the production of several oxygen-centered radicals associated with the enhancement of phase-I reactions [reviewed in Bost (27)] can determine "oxidative stress" contributing to the carcinogenic potential, particularly of nongenotoxic carcinogens such as many pesticides (26).

The experimental approach presented, by evaluating the effects of pesticides at different levels of the multistep carcinogenic process (e.g., initiation and promotion) is fundamental to achieving a more complete and accurate evaluation of the genotoxic/carcinogenic profile of a pesticide. The generation of data at significant biological end points contributes to the design of regulatory instruments through which risk to humans can be minimized. Knowledge of the genetic and biochemical damage at various organ sites can be used in assessing the probable risk of the chemical. An example is represented by the semiquantitative approach using categories of concern for both mutagenicity and carcinogenicity (2,3).

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